# Immunogenicity of VEGF peptide mimics

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with distinction* in Biological Sciences in the undergraduate colleges of The Ohio State University

by

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## ABSTRACT

VEGF (vascular endothelial growth factor) is a growth factor responsible for angiogenesis, which is the development of new blood vessels from preexisting ones. Oxygen and nutrients are supplied to tumors by blood vessels and tumor growth is greatly limited without angiogenic capabilities. Targeting VEGF is a promising strategy for cancer treatment and preventing metastasis. The focus of this project is to inhibit VEGF-VEGFR-2 (VEGF receptor 2) interaction high affinity by producing anti-peptide antibodies that will compete with VEGF for the binding region of VEGFR-2. VEGFR-2 has been shown to bind VEGF and stimulate angiogenesis, thereby resulting in increased tumor growth and metastasis. The hypothesis to be tested is whether these anti-peptide antibodies are able to recognize VEGFR-2 by specifically binding to the protein. The peptides were synthesized by solid phase peptide synthesis and binding was tested against antibodies produced in outbred rabbits that had been immunized with the peptide vaccine. Antibodies to the natural sequence (VEGF 102-122), its cyclic sequence (VEGF P3), and its retro-inverso sequence (VEGF P4) were tested against the peptides in both simple and competitive ELISA. In the case of immunization, the VEGF B-cell epitopes were co-linearly synthesized with the promiscuous T-helper epitope MVF, which is the measles virus fusion protein. The B-cell and T-cell epitopes were linked by a flexible four-residue turn (GPSL) linker which allows for each epitope to fold independently. Linked with the B-cell epitopes were MVF-VEGF 102-122, MVF-VEGF P3, and MVF-VEGF P4. The synthesized peptide vaccines were immunogenic and elicited antibody production by the

immune system, which successfully bind and recognize the peptide and the natural VEGF protein. The antibodies should be able to prevent growth of tumors dependent on VEGF and angiogenesis. Results from the competitive ELISA showed that VEGF peptide mimics were able to prevent the antibodies from binding the VEGF protein. These highly specific anti-peptide antibodies should have therapeutic potential in most cancers that are dependent on angiogenesis.

~Dedicated to my family ~

Dr. J. Michael, Beth, Monica, Megan, and Joseph Thuney

And

All my wonderful friends

Laurin Turowski, Adam Millat, Adam Wrobel, Andrew Faiella, Nick Capote, and

Mitch Appleman

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# CHAPTER 1

# INTRODUCTION

## 1.1 *Cancer Today*

Cancer kills more than 562,000 Americans every year. It is responsible for one out of every four deaths in the United States [1]. It is the second leading cause of death in the United States following heart disease (612,000 deaths). In 2007, 23.2% of all deaths in the United States were due to cancer (Table 1.1). Although cancer is prevalent with over 1.5 million new cases diagnosed each year, survival rates have increased significantly in the past 25 years (Table 1.2). The data suggest that current treatments are able to lower mortality amongst the patients that have been diagnosed with cancer. The available options currently include surgery, radiation therapy, and chemotherapy. These all have debilitating side effects and most of the chemotherapeutic agents have unacceptable safety profiles and are also expensive. The physical and financial burden from cancer is immense with total cancer costs exceeded $228 billion dollars in 2008.

*1.2 Current Cancer Treatment Methods*

Cancer is the uncontrolled growth of cells that continually divide with no regulation. Treatments for cancer include surgical removal, controlling or killing the cancerous cells. Chemotherapy, surgical resection, and radiation therapy are the most common options for cancer treatment.

**Chemotherapy**

Chemotherapy is the use of strong drugs to treat cancer. With more than 100 variations of chemotherapeutic drugs available, doctors are able to select the one that would best treat the specific condition. This treatment method is effective for slowing the cancer growth and killing cancer cells that may spread to other areas. Oftentimes multiple chemotherapeutic drugs are used in combination to better combat cancer, which is called combination chemotherapy. Although chemotherapy is effective in killing cancerous cells it also kills normal cells, which is a major side effect for the treatment method. Hair loss, bone marrow density changes, memory and emotional changes are experienced by patients undergoing chemotherapy.

**Radiation Therapy**

Radiation therapy is the use of high-energy particles, such as x-rays, gamma rays, electron beams, or protons, to destroy or damage cancer cells. It is used with other treatments or drugs known as radiosensitizers, which make the cancer cells more sensitive to the radiation. The therapy works by using concentrated radiation on the cancer cells to destroy their DNA and prevent growth, division, and spreading by the

cells. Normal cells in the area may also be affected, which is a side effect. Other side effects include radiation-induced nausea, skin changes, fatigue, and low blood counts.

**Surgery**

Surgery is a technique used to both diagnose and treat cancer. Cancer biopsies are tissue sections removed to screen for cancerous cells. Surgery is also used to remove tumors confined to one area. Although it is difficult to remove cancer tumors completely, the surgery in combination with other treatments can reduce the cancer greatly and increase survival rate. Side effects of surgery include excessive bleeding, complications during surgery including infection, pain, and damage to organs.

### *1.3 Function of VEGF*

VEGF, also called vascular permeability factor (VPF), is a pro-angiogenic factor and its actions are mediated through binding to two receptor tyrosine kinases, VEGFR-1 and VEGFR-2. By activating these receptors with VEGF, phosphorylation of proteins responsible for signal transduction for cellular regulation occurs [6]. VEGF plays a key role in development of the vascular network in vasculogenesis (embryogenesis or formation of new vessels) and angiogenesis (formation of new blood vessels from existing vessels) [7]. The VEGF protein family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor. Of these unique proteins, VEGF-A binds to VEGFR-1 and -2, which are involved in triggering angiogenesis, making it the most studied of the VEGF family [3].

*1.4 Tumors and Angiogenesis*

A major focus of the lab is the prevention of angiogenesis. Angiogenesis is the growth of new blood vessels from pre-existing vessels. The importance of inhibiting angiogenesis is seen in tumor suppression and cancer treatment. The tumors in multiple cancers have been shown to be dependant on tumor vasculature [2]. The process of angiogenesis is four fold: the degradation of the basal lamina that covers existing blood vessels, migration toward an angiogenic signal, proliferation and the eventual formation of new tubular structures [3]. Although increased angiogenesis has potential health benefits such as in a stroke or heart-attack patient, the proliferation of tumors due to angiogenic factors is a cause for concern. The tumors are able to maintain growth with sufficient oxygen, which is only possible with new vessels. With oxygen and essential nutrients being supplied, tumor growth is likely to occur. This process is driven by production of pro-angiogenic factors that override the effect of inhibitory angiogenic factors. One of these activators is VEGF (vascular endothelial growth factor) [4]. Because of noted dependence of tumor growth on angiogenesis, the idea of combining anti-angiogenic therapeutic drugs along with conventional cancer therapies has become the treatment option for most cancers today [4, 5].

*1.5 Anti-Angiogenic Agents as Therapy*

Chemotherapy has been the basis for cancer treatment for decades. This has presented considerable toxicities that limit the amount of treatment and cause differing adverse side effects in the patient as well as developments of immunogenicity to treatment. The development of specific agents that directly target the malignant cell or

tumor-supporting cells is being studied [6]. With the developing research on the role of angiogenesis and the dependence of growth on vascular supply, research targeting tumor angiogenesis has been a major focus in the past decade. Over-expression of VEGF has been seen in the majority of cancers, including colorectal, liver, lung, thyroid, breast, gastrointestinal tract, kidney, and bladder cancers as well as ovary and uterine cervix, among others [6]. One way to interrupt angiogenesis, directly or indirectly, depends primarily on interfering with the VEGF-dependent pathway, especially the VEGF: VEGFR-2 signaling.

Because of the dependence of tumor growth and metastasis on angiogenesis, anti-angiogenic therapies are being studied as alternatives in cancer treatment. Theoretically, starving a tumor of nutrients required for growth is possible by inhibiting the growth of new blood vessels to that tumor. A noted regulator of angiogenesis, which has led to tumor growth is seen in VEGF. As seen in preclinical and clinical studies, VEGF has a direct effect on tumor growth. The preclinical models showed greater growth and metastatic potential in human tumor xenografts, while clinical trials have shown early stages of breast cancer with associated VEGF expression have greater metastatic potential. The large impact VEGF and its receptors have had on angiogenesis has led to the focus of anti-angiogenic therapy by blocking these signals [2]. An example is Bevacizumab (Avastin®), a humanized monoclonal antibody developed by Genentech that was granted FDA approval as a drug to be used in colorectal cancer in 2005 and recently in breast cancer in 2008 [8,9]. It is so far the most successful anti-angiogenic inhibitor clinically, however some patients have demonstrated complications such as

hypertension, which may be due to VEGF and its role in the vascular system. Also cases of cardiotoxicity and resistance have been reported.

VEGFR-2 has demonstrated a highly active tyrosine kinase signal, which triggers angiogenesis. Inhibiting the kinase ATP binding sites is a greatly studied approach to prevent angiogenesis. The drugs are easily administered by oral doses. An issue in this treatment is seen when the kinase develops resistance by mutating [10,11]. Sunitinib (Sutent®), is an FDA approved tyrosine kinase inhibitor that targets VEGFR-2 and was approved in 2006 for first-line therapy for advanced renal cancer and gastrointestinal stromal tumor (GIST) [12].

*1.6 VEGF and its receptors*

The mechanism for VEGF receptor signaling comprises of receptor activation, by binding VEGF activation of the tyrosine kinase to phosphorylate proteins, and creation of docking sites for signaling proteins from several pathways. Although VEGF binds to a variety of cell surface proteins, the highest affinity is to VEGFR-1 and VEGFR-2, transmembrane receptor tyrosine kinases. These receptor proteins are expressed mainly in the endothelial cells of the vascular endothelium. Although VEGF binds to VEGFR-1 with higher affinity, the receptor activation is weaker for unknown reasons [3]. As a result of the weak receptor, it was thought that VEGFR-1 was simply a decoy to decrease VEGFR-2 signaling, but recent studies have shown the importance of VEGF-1 signaling in the activation of migration in the monocytes/macrophages, recruiting them to the tumor microenvironment [13, 14, 15]. It has been found that VEGF and VEGFR-2

interaction is required in order to trigger angiogenesis and the blocking of this interaction is a viable strategy for developing anti-angiogenic drugs [13, 16-21].

*1.7 Peptides and Retro-Inverso Peptides*

Peptides are small chains of amino acids that mimic parts of an entire protein. The site of interest that the peptide mimics is generally biologically active and accessible. Synthetic peptides are developed usually by using a structure-based design of the binding or active site that is trying to be inhibited (Figure 1.2). These peptides are chemically synthesized to fit the molecules as precisely as possible. The key factor when synthesizing peptides is designing them while maintaining conformational space and orientation of the bioactive surface while retaining sufficient flexibility to bind cooperatively with a given receptor. The therapeutic benefits of peptides include being water soluble, non-immunogenic, and able to easily cross tissue barriers. The drawback of using peptides as a therapeutic agent is that they are easily degraded *in vivo* by proteases, thereby requiring repeated treatments. In order to prevent degradation by proteases, retro-inverso modification is used. Retro-inverso modification is the reverse of the peptide backbone by inverting the amino acid sequence and chirality by utilizing D-amino acids (Figure 1.3). The retro-inverso peptides have the same orientation of the side chains, which appear topographically equivalent to the original peptide. Natural amino acids, easily recognized by the body are L-amino acids so the use of D-amino acids generally will avoid being degraded by proteases and make their use *in vivo* a better option (Table 1.3) [3].

*1.8 Vaccine Strategy using Peptides*

The role of antibodies is to specifically recognize and bind foreign antigens. B-cells produce antibodies and protection against repeated exposure against the antigens and this is seen because B-cell memory is activated when exposed again. The ability to produce antibodies outside the body and introduce them as therapeutic agents, offers a new alternative. An important component of administering the therapeutic antibodies is them not being recognized as a foreign antigen to the body when administered [26, 27]. A mechanism of action by these antibodies is the interaction protein-protein interaction by specifically targeting the receptor site where the binding occurs [28].

High affinity antibodies need to be able to bind to epitopes that contain essential residues for their interaction. The epitopes need to be designed, taking into consideration that the binding area of a protein is the result of a structural arrangement of residues. Peptides can also combine both B- and T-cell epitopes constructed in a collinear fashion. Using a universal T-helper epitope from measles virus fusion protein (MVF), the immunogenicity of designed peptides is increase [29, 30]. Because VEGF 102-122, VEGF P3, and VEGF P4 are all B-cell epitopes, a MVF sequence was linked to them to evaluate the potential to generate antibodies that would recognize and specifically bind VEGF. These anti-VEGF peptide mimic antibodies should show inhibitory effects due to binding to VEGF and the blockage of the VEGF-VEGFR-2 interaction.

*Figures*

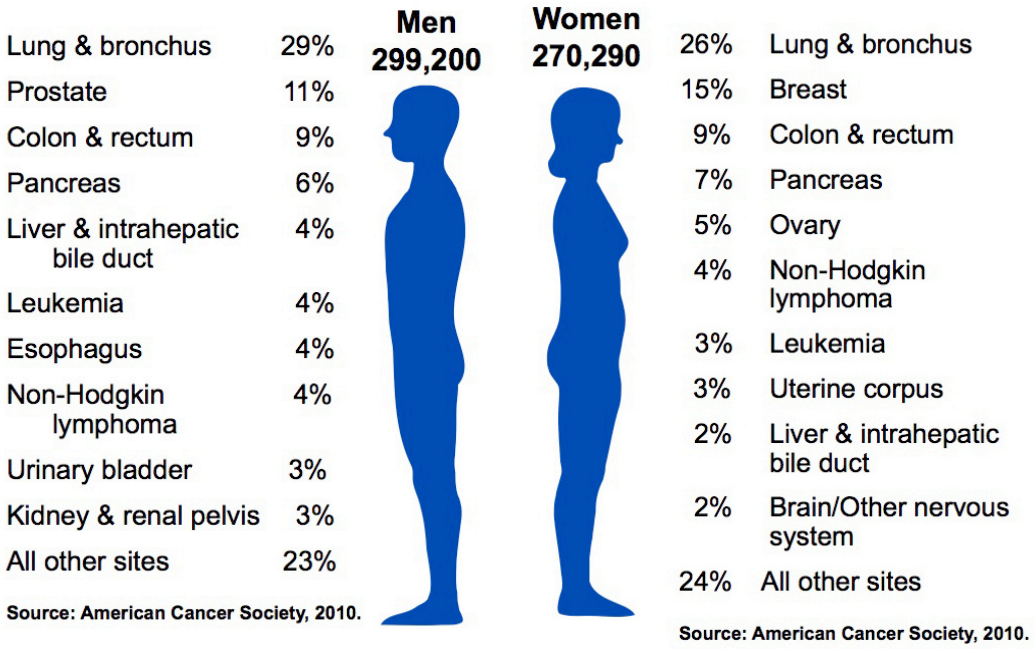



**Table 1.1** Number of cancer related deaths in 2007 in the United States. VEGF is over expressed in many of these cancers.

Trends in Five-year Relative Survival (%)* Rates, US, 1975-2005

| Site | 1975-1977 | 1984-1986 | 1999-2005 |
|---|---|---|---|
| All sites | 50 | 54 | 68 |
| Breast (female) | 75 | 79 | 90 |
| Colon | 52 | 59 | 66 |
| Leukemia | 35 | 42 | 54 |
| Lung and bronchus | 13 | 13 | 16 |
| Melanoma | 82 | 87 | 93 |
| Non-Hodgkin lymphoma | 48 | 53 | 69 |
| Ovary | 37 | 40 | 46 |
| Pancreas | 3 | 3 | 6 |
| Prostate | 69 | 76 | 100 |
| Rectum | 49 | 57 | 69 |
| Urinary bladder | 74 | 78 | 82 |

***5-year relative survival rates based on follow up of patients through 2006.**
**Source: Surveillance, Epidemiology, and End Results Program, 1975-2006, Division of Cancer Control and Population Sciences, National Cancer Institute, 2009.**

**Table 1.2** Trends in survival rate of diagnosed cancers between 1975 and 2005.

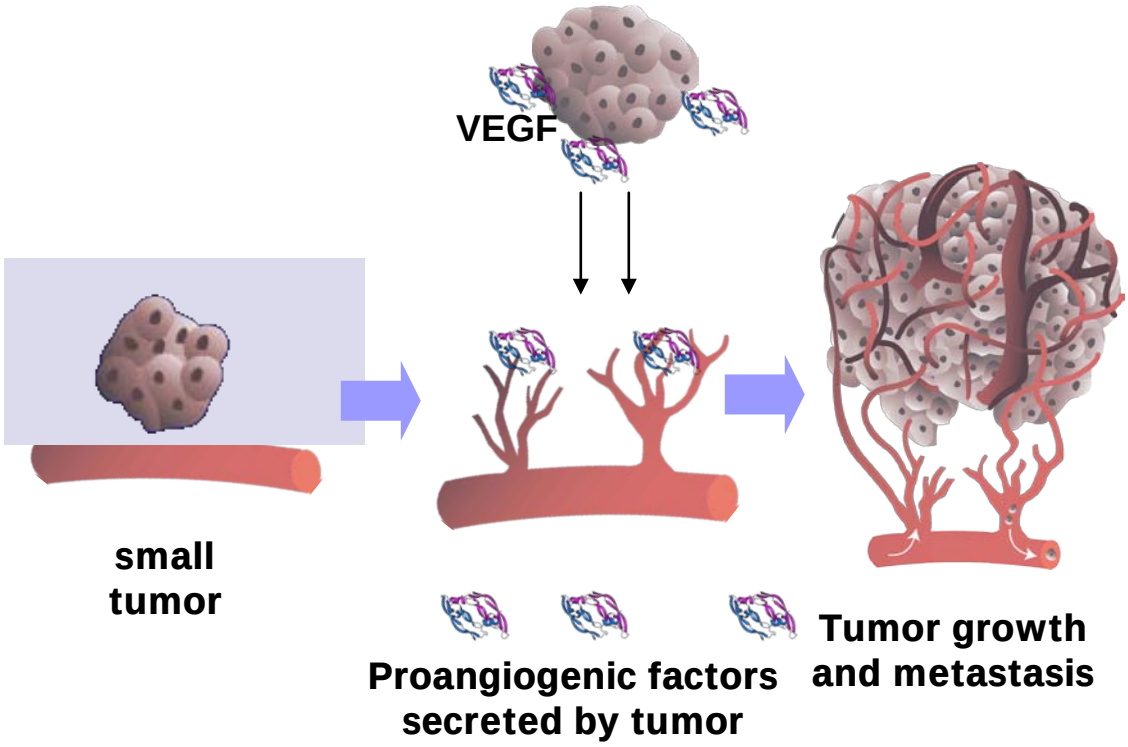



**Figure 1.1 Angiogenesis dependent tumor growth and metastasis.** Tumor cells up-regulate pro-angiogenic factors (VEGF) that promote the formation of new blood vessels to supply tumors with nutrients and oxygen needed to grow. After certain growth, the tumors metastasize and use the vessels to migrate to new sites throughout the body.

| peptides: Peptide | Sequence | #AA | Mol. Wt. (Da) |
|---|---|---|---|
| VEGF 102-122 | (102)-76-ITMQ IMRIKPHQGQHIG EMSF-96-(122) | 21 | 2481 |
| VEGF-P3(CYC) | 76–ITMQ-79-C-92-GIHQGQHPKIRMI-80-C-EMSF-96 | 23 | 2724 |
| VEGF-P4(CYC) | 76-ITMQ-79-C-92-IMRIKPHQGQHIG-80-C-EMSF-96 | 23 | 2724 |

**Table 1.3 Amino acid sequences and molecular weight of VEGF peptide mimics.** Sequences of amino acids are represented from N to C-terminal. #AA represents number of amino acids in each peptide and Mol. Wt. represents the molecular weight of peptides.

A. B.

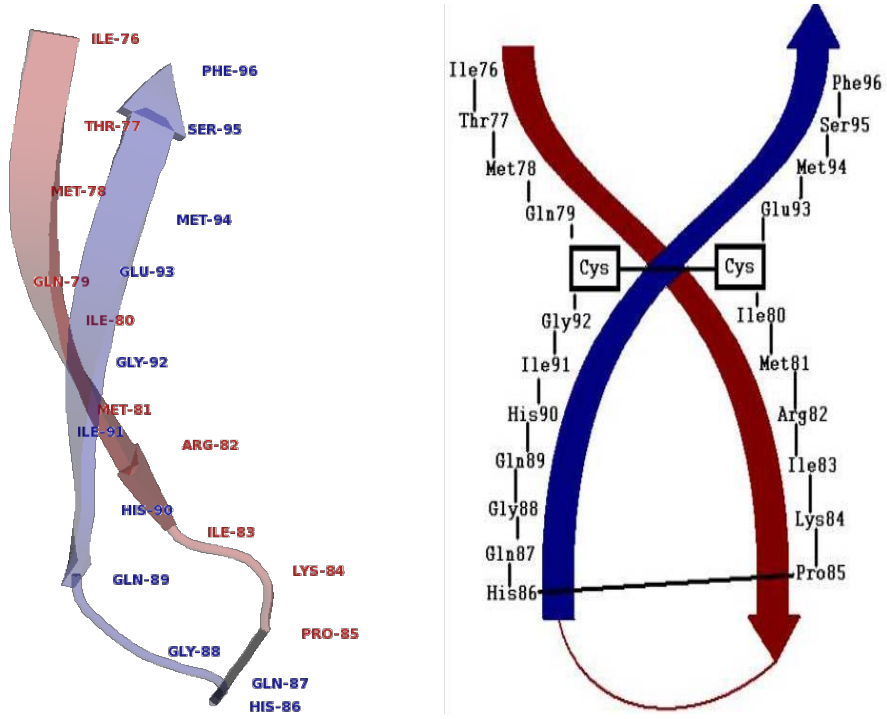



***Figure 1.2***: **Schematic representation of the VEGF peptide mimic design.** (**A**) VEGF sequence 102-122 (76-96) from crystal structure with labeled residues. (**B**) Peptide mimic VEGF-P3(CYC). In black is shown the VEGF-P3(CYC) peptide sequence with labeled residues; arrows show anti-parallel β-sheet orientation based on the crystal structure as represented in (A) with the same colors.

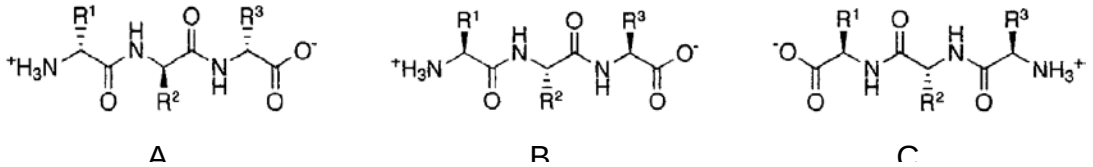



***Figure 1.3*: Schematic representation of the effects of retro-inverso peptides .** Natural orientation of parent L-amino acid peptide (**A**). Reversed side chain orientation in D-amino acid peptide (**B**). Retro-inverso peptide with restored side chain orientation (**C**).

# CHAPTER 2

# METHODS AND MATERIALS

## *2.1 Peptide Synthesis*

Peptides were synthesized on Milligen/Biosearch 9600 solid-phase peptide syntesizer using Fmoc/t-But chemistry. VEGF 102-122 was synthesized using preloaded Fmoc-Phe-CLEAR acid resin while VEGF-P3 was synthesized using CLEAR amide resin (Pepides International, Louisville, KY). VEGF-P3 was acetylated on resin, using acetyl imddazole reagent following protocol. All peptides were cleaved from the resin using cleavage reagent B (Trifluoroacteic acid:Phenol:Water:Triisopropyl silane 90:4:4:2) and crude peptides were purified on preparative RP-HPLC (Reverse Phase-High Pressure Liquid Chromatography) using Vydac C-4 column and acetonitrile-water (0.1 % TFA) gradient system. All fractions were analyzed on analytical RP-HPLC and characterized by MALDI (Matrix Assisted Laser Desorption Ionization mass spectroscopy) at CCIC (Campus Chemical Instrumentation Center, The Ohio State University, Columbus, OH). RP-HPLC pure peptide VEGF-P3 containing two Cysteine residues was synthesized retro-inverso to form VEGF-P4 (RI-VEGF-P3). Amino acid sequences and molecular weight of VEGF peptide mimics as determined by MS are shown in Table 1.1.

2.2 *Rabbit Immunization*

New Zealand white rabbits were immunized with 1 mg of peptide dissolved in distilled water emulsified (1:1) in Montanide ISA720 vehicle (Sepic) with 100 µg of *N*-acetylglucosamine-2yl-acetyl-1-alanyl-d-isoglutamine (nor-MDP). Rabbits were boosted with the respective doses at three week intervals. Rabbit blood was collected via the central auricular artery and sera were tested for antibody titers. Anti-peptide antibodies were purified by affinity chromatography using a Protein A/G column (Thermo Fisher Scientific Inc, Rockford, IL) from high-titer sera.

2.3 *Antibody Purification*

Sear, 3 mL was diluted a minimum of 1:1 using de-gassed Binding Buffer. The sodium azide storage solution was allowed to run through the column. Twice, Binding Buffer was equilibrated running 10 mL through and the pH was checked to be approximately 8.0. The diluted sample was allowed to run before washing the column using 20 mL of Binding Buffer. The antibodies from the column were eluted by using 10 mL of Elution Buffer. Ten 1 mL fractions were collected in pre-labeled eppendorf tubes after 10 mL of buffer were added. The column was regenerated by running 8 mL of Elution Buffer through it. The samples of eluted antibodies were read at 280 nm using a Spectrophotometer. The samples that have an absorbance reading of 0.3 or higher are pooled and spun down in a filter centrifugation tube at 2000 rpm for 30 min. The samples were topped with 4 mL of 0.1% Tween 20/PBS and after 2 washes the concentrated

antibody was transferred to a labeled eppendorf tube where using a Coomassie Plus Protein Assay, the concentration of the antibody were determined.

2.3 *VEGF Direct ELISA*

Plates were coated overnight at 4 °C with 100 uL of 2 ug/mL rhVEGF (R&D Systems, Minneapolis, MN), washed four times with 0.1 % Tween 20/PBS, and blocked with 200 uL of 1% BSA/PBS for 1 h at room temperature. Plates were washed four times with 0.1% Tween 20/PBS. Anti-peptide sera were serially diluted down the plate to cerate several dilutions and incubated 2 h at room temperature. Plates were washed four times with 0.1 % Tween 20/PBS, a 1/500 dilution of goat-anti-rabbit IgG HRP was added and incubated 1 h. Detection was done coating the plate with ABTS substrate in the dark for 10 minutes before absorbance reading took place at 415 nm.

2.4 *Competitive ELISA*

Plates were coated overnight at 4 °C with 100 uL of 2 ug/mL rhVEGF (R&D Systems, Minneapolis, MN), washed four times with 0.1 % Tween 20/PBS, and blocked with 200 uL of 1% BSA/PBS for 1 h at room temperature. Plates were washed four times with 0.1% Tween 20/PBS. 50 uL of PBT/HS were added to rows B through H and 100 uL of 40uM of soluble inhibitor added to top row where it was serially diluted down the column except last wells. Sera dilutions based on direct ELISA titer where dilution curve is linear. 50uL of sera (antibody) dilution added to each well. Plates incubated at room temperature for two hours. Plates were washed four times with 0.1 % Tween 20/PBS, a

1/500 dilution of goat-anti-rabbit IgG HRP was added and incubated 1 h. Detection was using ABTS substrate was read at 415 nm.

# CHAPTER 3

# RESULTS

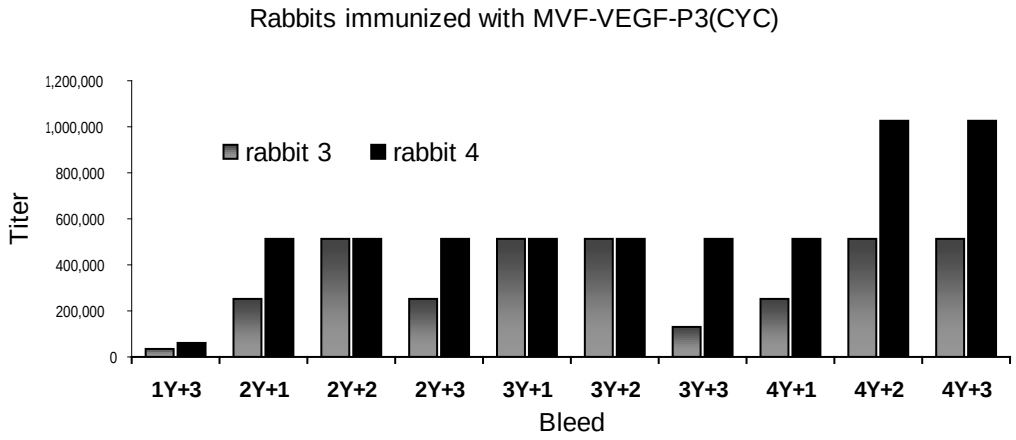



**Figure 3.1 Antibody responses elicited by MVF-VEGF-P3 peptide vaccines in outbred rabbits.** Two rabbits per group were each immunized with the MVF-VEGF-P3(CYC) peptide. Blood was drawn weekly, and sera surveyed for peptide-specific antibody by ELISA. The results of each individual rabbit are shown. Titers are defined as the reciprocal of the highest serum dilution with an absorbance of 0.2 or greater after subtracting the background. 4y+3w indicates the antibody titer in blood drawn three weeks after the fourth immunization [1].

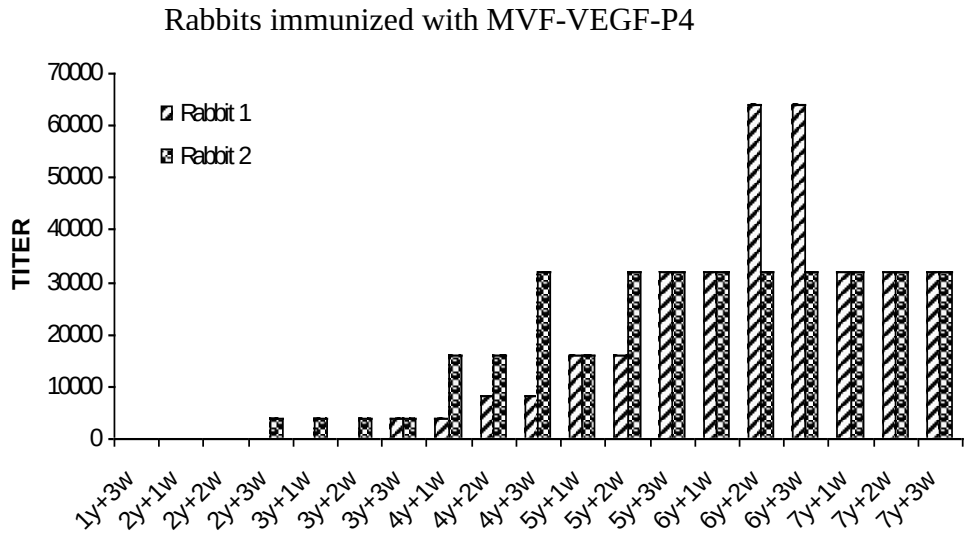
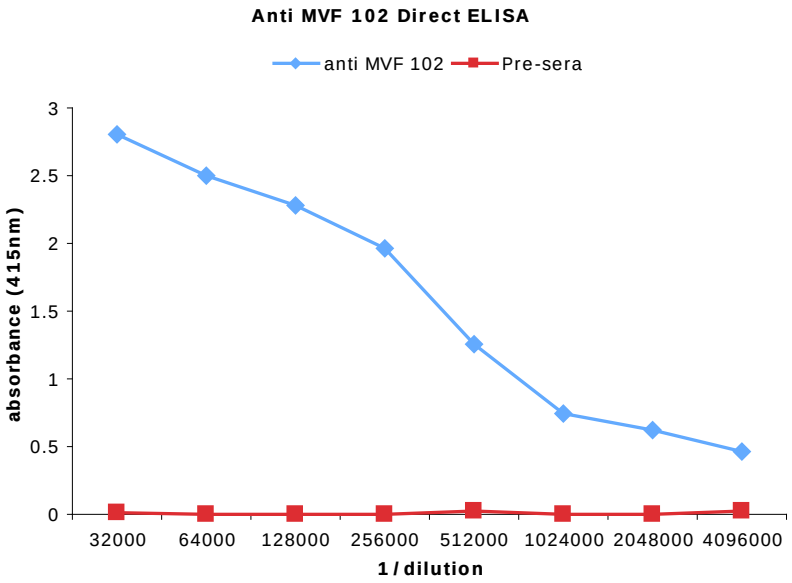



**Figure 3.2 Antibody responses elicited by MVF-VEGF-P4 peptide vaccines in outbred rabbits.** Two rabbits per group were each immunized with the MVF-VEGF-P3 peptide. Blood was drawn weekly, and sera surveyed for peptide-specific antibody by ELISA. The results of each individual rabbit are shown. Titers are defined as the reciprocal of the highest serum dilution with an absorbance of 0.2 or greater after subtracting the background. 4y+3w indicates the antibody titer in blood drawn three weeks after the fourth immunization [1].

A

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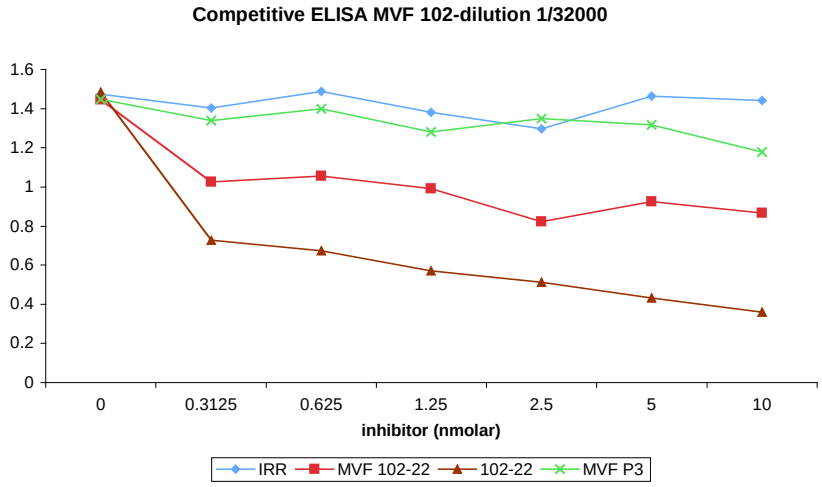
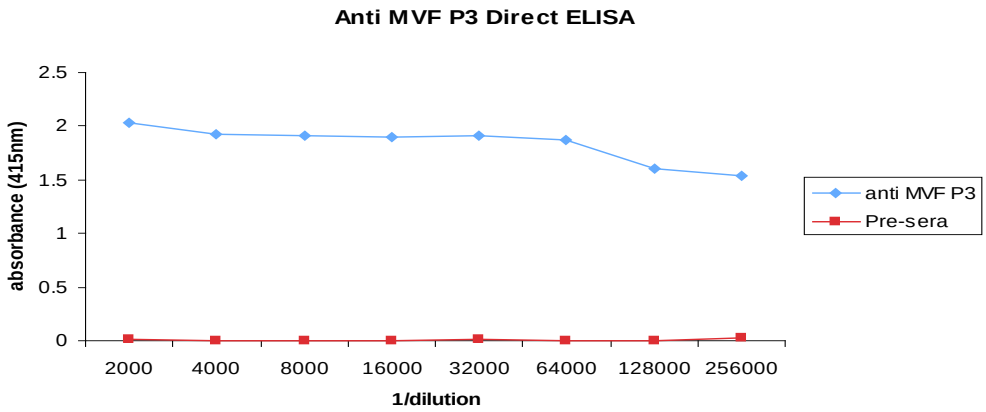



**Figure 3.3 Anti-MVF-VEGF 102-122 competitive inhibition ELISA.** Plates were coated with MVF-VEGF 102-122 peptide and anti-MVF-VEGF 102-122 binding was analyzed directly (**A**) or in competition assay (**B**) where VEGF peptide mimics were used as inhibitor.

Figure 3.3 shows the direct (A) and competitive ELISA (B) between MVF-VEGF 102-122 and anti-MVF-VEGF 102-122 antibodies, which were produced after New Zealand white rabbits were immunized three times at three-week intervals. The bleed, 4y+1w (one week after the fourth immunization) was found to have the highest titer readings. The purified antibodies from the 4y+1w bleed were then added to the 96-well plates that had been coated with MVF-VEGF 102-122. The competitive ELISA also used different peptides as inhibitors to prevent binding between MVF-VEGF 102-122 and its antibodies. As is expected, the irrelevant peptide (IRR) showed no ability to inhibit MVF-VEGF 102-122 binding to its antibody. The peptide has an amino acid sequence that does not correspond to the VEGF-VEGFR-2 sequence and therefore it should show no inhibition of binding .MVF-VEGF P3, VEGF 102-122, and MVF-VEGF 102-122 had different binding inhibiting abilities.

A




B

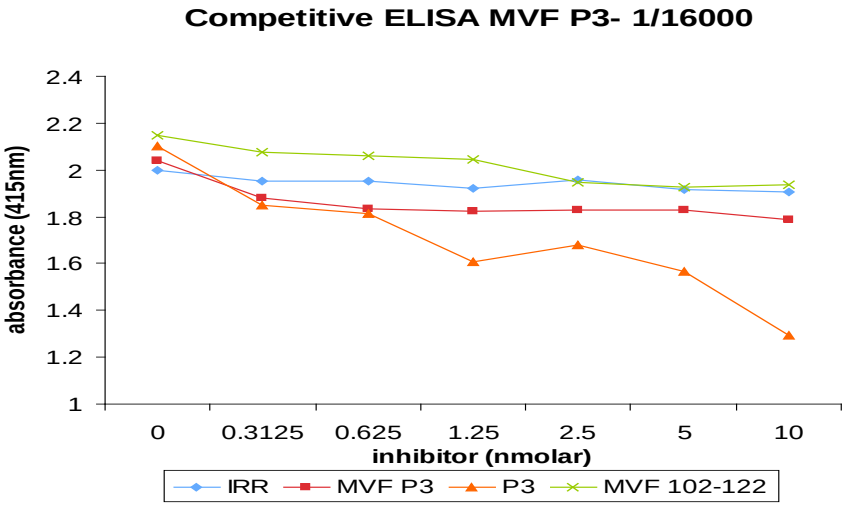
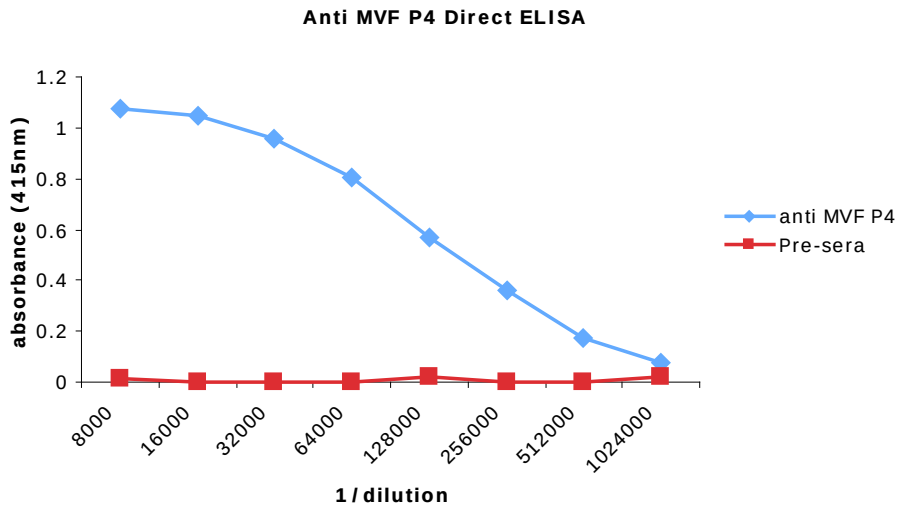



**Figure 3.4 Anti-MVF-VEGF P3 competitive inhibition ELISA.** Plates were coated with MVF-VEGF P3 peptide and anti-MVF-VEGF P3 binding was analyzed directly (**A**) or in competition assay (**B**) where VEGF peptide mimics were used as inhibitors.

Figure 3.4 shows the direct (A) and competitive ELISA (B) between MVF-VEGF P3 and anti-MVF-VEGF P3 antibodies, which were produced after New Zealand white rabbits were immunized three times at three week intervals. The bleed, 4y+3w (three weeks after the fourth immunization) was found to have the highest titer readings (Figure 3.1). The purified antibodies from the 4y+3w bleed were then added to the 96-well plates that had been coated with MVF-VEGF P3. The competitive ELISA also used different peptides as inhibitors to prevent binding between MVF-VEGF P3 to its antibody. The irrelevant peptide (IRR) showed no ability to inhibit MVF-VEGF P3 binding to its antibody. MVF-VEGF P3, VEGF 102-122, and MVF-VEGF 102-122 all had differing biding inhibition abilities with VEGF P3 showing the most inhibition.

A

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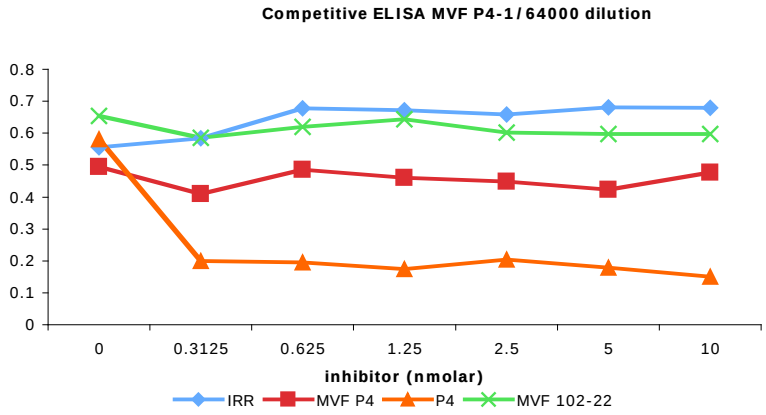
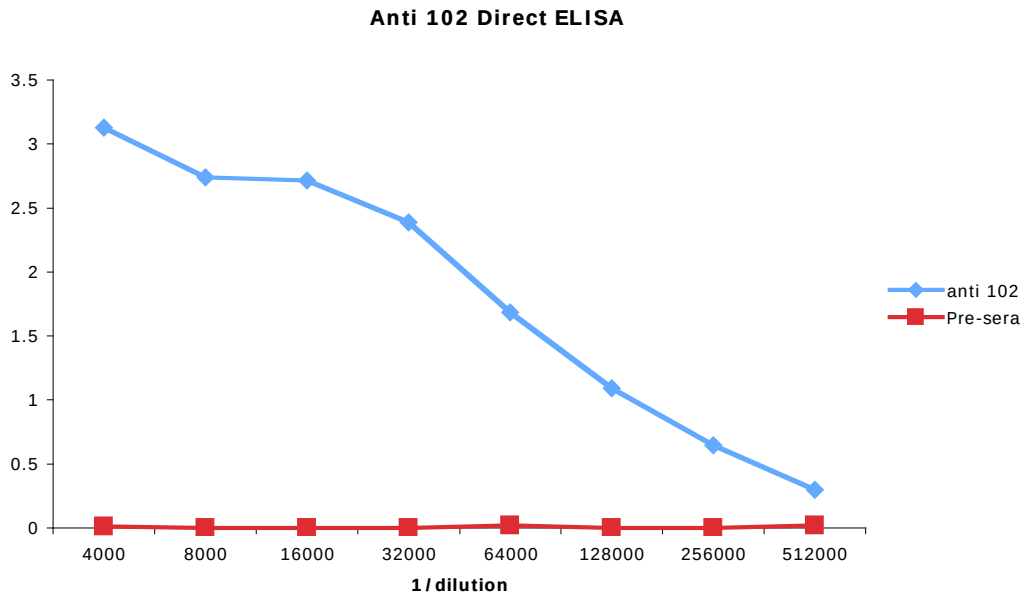



**Figure 3.5 Anti-MVF-VEGF P4 competitive inhibition ELISA.** Plates were coated with MVF-VEGF P4 peptide and anti-MVF-VEGF P4 binding was analyzed directly (**A**) or in competition assay (**B**) where VEGF peptide mimics were used as inhibitors.

Figure 3.5 shows the direct (A) and competitive ELISA (B) between MVF-VEGF P4 and anti-MVF-VEGF P4 antibodies, which were produced after New Zealand white rabbits were immunized three times at three-week intervals. The bleed, 6y+3w (three weeks after the sixth immunization) was found to have the highest titer readings (Figure 3.2). The purified antibodies from the 6y+3w bleed were then added to the 96-well plates that had been coated with MVF-VEGF P4. The competitive ELISA also used different peptides as inhibitors to prevent binding between MVF-VEGF P4 and its antibodies. The irrelevant peptide (IRR) showed no ability to inhibit MVF-VEGF P4 binding to its antibody. MVF-VEGF P4, VEGF P4, and MVF-VEGF 102-122 had differing binding inhibiting abilities with VEGF P4 having the greatest inhibiting abilities.

A




B

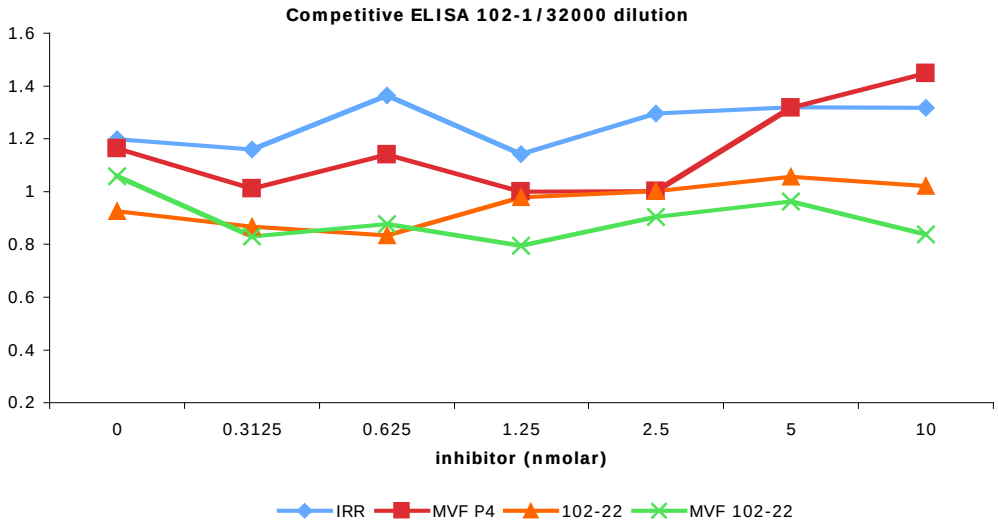
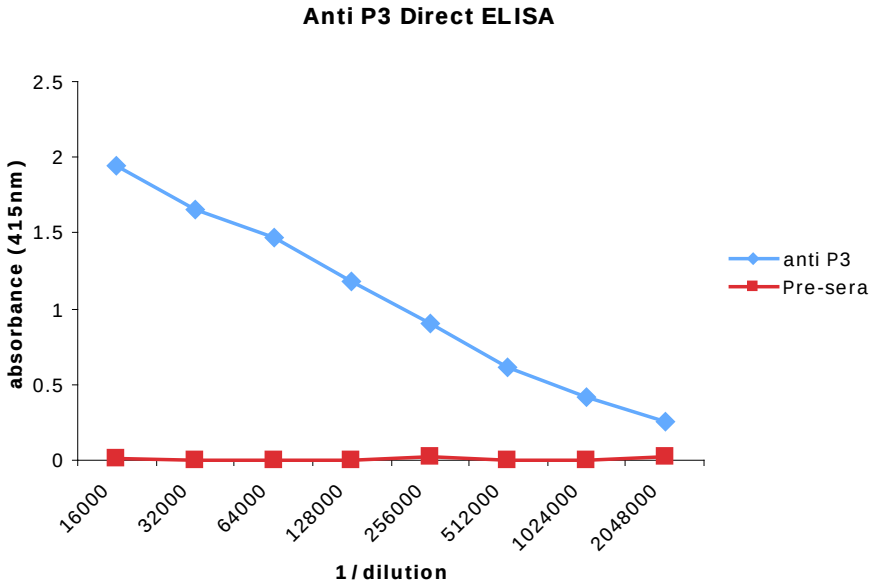



**Figure 3.6 Anti-VEGF 102-122 competitive inhibition ELISA.** Plates were coated with VEGF 102-122 peptide and anti-VEGF 102-122 binding was analyzed directly (**A**) or in competition assay (**B**) where VEGF peptide mimics were used as inhibitors.

Figure 3.6 shows the direct (A) and competitive ELISA (B) between VEGF 102-122 and anti- VEGF 102-122 antibodies, which were produced after New Zealand white rabbits were immunized three times at three week intervals. The bleed, 4y+1w (one week after the fourth immunization) was found to have the highest titer readings. The purified antibodies from the 4y+1w bleed were then added to the 96-well plates that had been coated with VEGF 102-122. The competitive ELISA also used different peptides as inhibitors to prevent binding between VEGF 102-122 and its antibodies. As is expected, the irrelevant peptide (IRR) showed no ability to inhibit VEGF 102-122 binding to its antibody. The peptide has an amino acid sequence that does not correspond to the VEGF-VEGFR-2 sequence and therefore it should show no inhibition. MVF-VEGF P4, VEGF 102-122, and MVF-VEGF 102-122 had inconsistent inhibiting abilities.

A




B

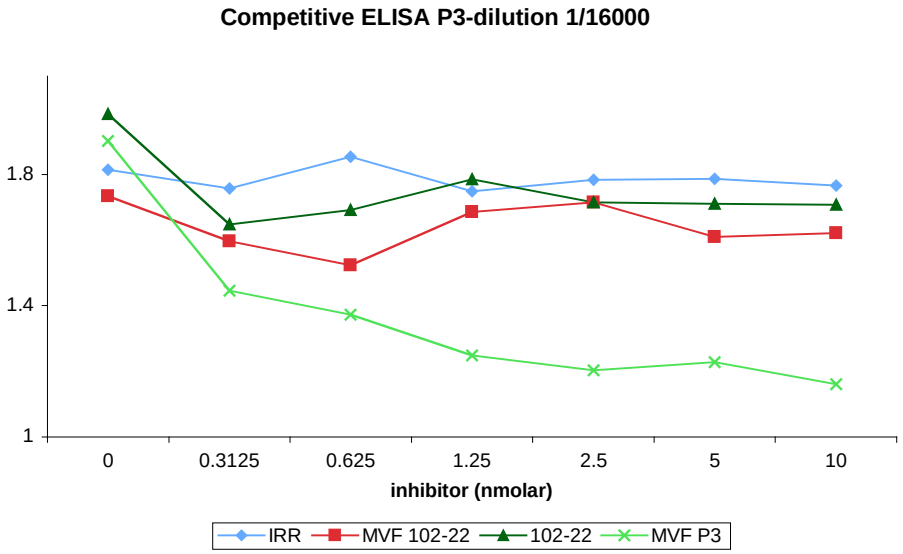
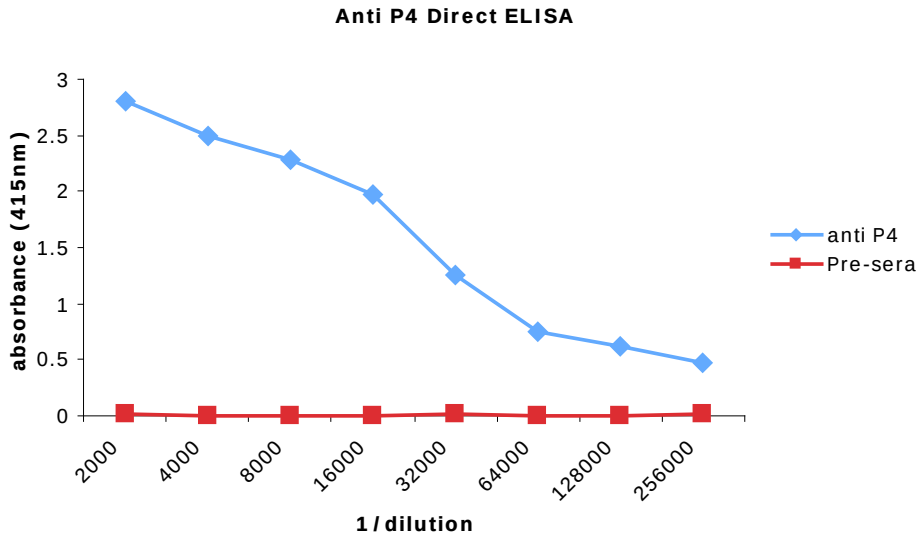



**Figure 3.7 Anti-VEGF P3 competitive inhibition ELISA.** Plates were coated with VEGF P3 peptide and anti -VEGF P3 binding was analyzed directly (**A**) or in competition assay (**B**) where VEGF peptide mimics were used as inhibitors.

Figure 3.7 shows the direct (A) and competitive ELISA (B) between VEGF P3 and anti-VEGF P3 antibodies, which were produced after New Zealand white rabbits were immunized three times at three-week intervals. The bleed, 4y+3w (three weeks after the fourth immunization) was found to have the highest titer readings (Figure 3.7). The purified antibodies from the 4y+3w bleed were then added to the 96-well plates that had been coated with VEGF P3. The competitive ELISA also used different peptides as inhibitors to prevent binding between VEGF P3 and its antibodies. As is expected, the irrelevant peptide (IRR) showed no ability to inhibit VEGF P3 to its antibody. The peptide has an amino acid sequence that does not correspond to the VEGF-VEGFR-2 sequence and therefore it should show no inhibition. MVF-VEGF P3, VEGF 102-122, and MVF-VEGF 102-122 all had similar inhibiting abilities with MVF-VEGF P3 showing a decrease in binding of the VEGF P3 peptide to the anti-VEGF P3 antibody.

A




B

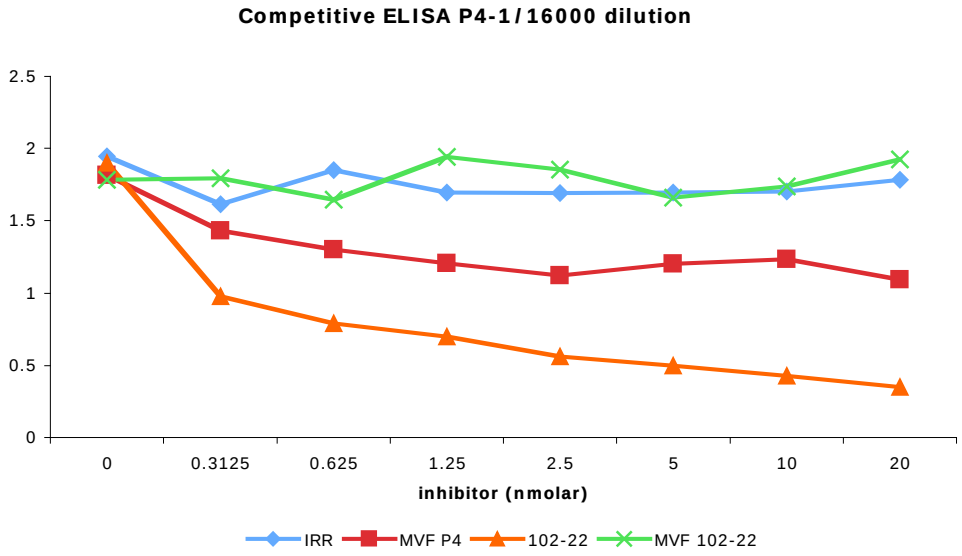



**Figure 3.8 Anti-VEGF P4 competitive inhibition ELISA.** Plates were coated with VEGF P4 peptide and anti-VEGF P4 binding was analyzed directly (**A**) or in competition assay (**B**) where VEGF peptide mimics were used as inhibitors.

Figure 3.8 shows the direct (A) and competitive ELISA (B) between VEGF P4 and anti-VEGF P4 antibodies, which were produced after New Zealand white rabbits were immunized three times at three week intervals. The bleed, 6y+3w (three weeks after the sixth immunization) was found to have the highest titer readings (Figure 3.8). The purified antibodies from the 6y+3w bleed were then added to the 96-well plates that had been coated with VEGF P4. The competitive ELISA also used different peptides as inhibitors to prevent binding between VEGF P4 and its antibodies. As is expected, the irrelevant peptide (IRR) showed no ability to inhibit VEGF P4 binding to its antibody. The peptide has an amino acid sequence that does not correspond to the VEGF-VEGFR-2 sequence and therefore it should show no inhibition. VEGF P4, VEGF102-122, and MVF-VEGF 102-122 had differing inhibiting abilities.

# CHAPTER 4

# DISCUSSION

In each competitive ELISA the antibodies with a specific dilution that was based on the linear portion of the direct ELISA was used. At the point on the direct ELISA where the graph is linear, the dilution at that value is considered an ideal control for competitive ELISA using antibodies at that dilution. The results showed that the antibodies produced in rabbits after peptide vaccine immunization were able to recognize and bind the VEGF protein. The peptides were also able to compete with VEGF for binding to the antibodies. In figure 3.3, the direct ELISA showed a gradual change in binding as the antibodies were diluted. In the competitive ELISA, the irrelevant peptide was not able to inhibit binding while the VEGF 102-122 peptide showed the most effects due to its similar structure with the coated protein, allowing for inhibition to occur. MVF-VEGF P3 showed very little inhibiting ability. VEGF 102-122 showed the most inhibition followed by MVF-VEGF 102-122 which are both expected because they share exact sequences and conformational structures as the peptide they are competing against the antibodies for binding.

In figure 3.4, the direct ELISA does not decrease as expected. The high binding made choosing a dilution for the competitive ELISA, inaccurate. In the competitive

ELISA, the irrelevant peptide was not able to inhibit binding while the VEGF P3 peptide showed the most effects due to its similar structure with the coated protein, allowing for inhibition to occur. MVF-VEGF P3 and MVF-VEGF 102-122 showed very little inhibiting ability. The high binding signifies little inhibition by peptides and high binding affinity of the antibody to the peptides. This may be due to the inhibiting peptides being denatured after numerous thawing and re-freezing which can take its toll on the structural integrity of peptides. Each competitive ELISA should show nearly identical binding for the wells with no inhibiting peptide. This is because no peptide is competing and is a measure of the consistency of the tests. As the inhibiting peptides concentration increases, the MVF P3, MVF 102-122, and 102-122 peptides are expected to show different inhibiting abilities depending on what peptide is coated on the plate that they are competing with the antibodies for the binding region of the peptide.

In figure 3.5, the direct ELISA showed a gradual change in binding as the antibody concentration was diluted. The competitive ELISA, showed little binding inhibition in the irrelevant peptide, and a gradual decrease in each of the other peptides with VEGF P4 showing the most change due to its similar structure with the coated protein, followed by MVF-VEGF P4, allowing for inhibition to occur. VEGF P4 is observed to be the best competing peptide for MVF-VEGF P4. The peptide is observed to be inhibiting on the antibody-peptide interactions and would be a good candidate for *in vivo* trials.

Figure 3.6 had a direct ELISA that showed a gradual change in the binding as the antibodies were diluted. In the competitive ELISA, the irrelevant peptide was not able to inhibit binding while the VEGF 102-122 peptide showed the most change due to its

similar structure with the coated protein, allowing for inhibition to occur. No peptide showed significant inhibiting abilities or a greater decrease in binding between the VEGF 102-122 peptide and its antibody should have been observed via the absorbance readings decreasing as inhibitor concentration increased. This may be due to degradation of the peptide mimics after a number of tests had been conducted with them. The results could be clarified with more trials and consistent data that was observed time and time again using newly synthesized peptide mimics.

As seen in figure 3.7, the direct ELISA had decreased binding as the antibodies were diluted. The competitive ELISA, showed inhibition in binding when irrelevant peptide was competing and a gradual decrease in binding was seen in each of the other peptides with the MVF-VEGF P3 peptide showing the most inhibition. It is expected that MVF-VEGF P3 has the greatest inhibition. MVF-VEGF 102-122 and VEGF 102-122 show similar binding inhibition, which is also expected as both peptides have the same B-cell epitope, which is not cyclized and therefore not a conformational match for the VEGF P3, coated on the plate.

Figure 3.8 has a direct ELISA that showed decreased antibody binding as the antibodies were diluted. In the competitive ELISA, the irrelevant peptide was not able to inhibit binding while the VEGF 102-122 peptide showed the most inhibition followed by MVF-VEGF P4. VEGF 102-122 has a more flexible structure due to it not being cyclized. As a result, *in vitro* tests may yield higher results compared to cyclized peptides (VEGF P3 and VEGF P4), but *in vivo* testing may show differing affinities.

# CHAPTER 5

## CONCLUSION AND CONTINUING RESEARCH

As more and more research is conducted on cancer and as drug therapies are being developed for the prevention of continued tumor growth and metastasis, the inhibition of the tumor growth has become a highly studied aspect by most researchers. The cost to develop novelty drugs is very expensive as the process includes discovery and implementation of the drugs with patents increasing the already taxing cost. Because of financial questions, companies and researchers have turned to the idea of peptide use to combat tumor growth by inhibiting receptor sites [25]. The focus of the research was on the interaction between an over expressed oncoprotein, VEGF, and its receptor site seen on tumors where it signaled angiogenesis to begin.

The focus of the research was on the efficacy of the VEGF peptide vaccine in producing antibodies that can inhibit the interaction between VEGF and VEGFR-2. The ELISA tests are effective in showing *in vitro* that the RI-VEGF peptide sequences can inhibit the binding of the anti-VEGF antibodies. This knowledge can be applied to animal and eventually human trials. Continued studies of VEGF's role in angiogenesis are being looked at and currently, research is looking at the combination of peptide mimics for VEGFR-2 with other peptide mimics for alternative growth mechanisms. The use of

synthetic peptide vaccines that target VEGFR-2 is an important component to cancer therapy.

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